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Original Research Article

Phytochemistry of *Syzygium polycephalum*

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ABSTRACT

Syzygium polycephalum is a plant that grows in several parts of Indonesia and belongs to Myrtaceae family. This research aims to reveal the isolation and structure elucidation of the bioactive compounds in this plant's fruit, seeds and leaves as part of overall research on the genus *Syzygium*. The isolation process included extraction, separation and purification, while the structure elucidation of isolated compounds was executed using the spectroscopic method. As a result, 1,5-dimethylcitrate and trimethyl citrate were isolated from the dichloromethane extract of the fruit, while from the ethyl acetate extract of the seeds, methyl gallate and gallic acid were isolated and identified. From the dichloromethane extract of the leaves, 3,4,3'-tri-*O*-methylellagic acid was isolated and identified. Determination of antioxidant activity was executed using the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) approach, with Trolox as a standard. Gallic acid showed the best antioxidant activity among the five isolated compounds with an IC_{50} of 29.6 g/mL. From this study, five secondary metabolites were isolated. Some showed strong activity and some were inactive as antioxidant.

Keywords: ABTS, Antioxidant, Myrtaceae, Phenolic compounds, *Syzygium polycephalum*.

Introduction

Syzygium polycephalum, a plant belonging to the Myrtaceae family, is widely distributed in Kalimantan and Java islands in Indonesia. This plant is locally known as gowok, kupa, kepa, or ruruhi. Ethnobotanically, the bark of this plant is consumed by decocting for the traditional treatment of dysentery.[1] Several phytochemical studies have been conducted on this plant. The whole fruit was reported to contain anthocyanin with strong antioxidant activity.[2] The ethanol extract of the leaves also showed intense antioxidant activity (IC_{50} of 10.327 ppm), although less active than ascorbic acid.[3] The antioxidant activity of the cortex's ethanol and ethyl acetate fractions was significantly correlated with the IC_{50} of glucosidase inhibition and total phenolic compounds (TPC) and total flavonoid compounds (TFC) contained in each extract.[4] The methanol extract of the stem bark has also shown strong antioxidant activity.[5]
Two phenolic compounds, gallic acid and ellagic acid derivative, namely 3,4,3'-tri-*O*-methylellagic acid, were isolated from the chloroform fraction of the stem bark of *S. polycephalum*. The last compound showed antioxidant activity with an IC_{50} of 72.1 µg/mL (IC_{50} of ascorbic acid as a positive control was 11.5 µg/mL).[6,7] Two saturated fatty acid compounds from the leaves, particularly hexadecanoic acid and 3-ethylpropanoate, were successfully isolated and identified.[8] The ethyl acetate extract of *S. polychephalum* heartwood contained a secondary metabolite compound 3-*O*-glucosyl-3',4'5-trihydroxyflavonol with antifungal activity against wood-destroying fungi *Schizophyllum commune* and *Pleurotus* sp.[9]

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The isolation of ursolic acid, oleanolic acid, squalene, and *β*-sitosterol from the dichloromethane fraction of *S. polycephalum* leaves was also reported.[10]
Due to the limited reports of compounds contained in each part of this plant, a research aimed to reveal the isolation and structure elucidation of the bioactive compounds in this plant was needed. The present study reports an exploration of some parts of this plant, such as fruit, seeds and leaves. Five compounds were isolated from this study, some compounds showed good antioxidant activity by the ABTS method, and some had no activity.

Materials and Methods

Plant material
S. polycephalum fruits, seeds, and leaves were collected from the Lawu Mount, Jogorogo-Ngawi, in East Java, Indonesia. Plant samples were taken at an altitude of 917 MASL in March 2020. The plant material was identified at the Department of Biology, Faculty of Science and Technology, Universitas Airlangga. The voucher specimen (UA-MSp010320) was deposited at the Herbarium of Universitas Airlangga, Laboratory of Biosystematics, Department of Biology, Faculty of Science and Technology, Universitas Airlangga.

General experimental procedures
Precoated silica gel 60 F254 (25 sheets of 20 x 20 cm aluminium, Merck) was used for TLC (thin layer chromatography) analysis. Vacuum Liquid Chromatography (VLC) was executed utilizing silica gel GF254 Merck 60 (3.5–25.0 m). Silica gel 60 (700-200 mesh ASTM) was used in column chromatography (CC). The UV spectrum was measured on a UV-visible spectrometer (Shimadzu). FTIR (Shimadzu IRTracer-100) was used for the IR spectrum and values were recorded in cm^{-1}. ^{1}H and ^{13}C-NMR (Nuclear Magnetic Resonance) were recorded with BRUKER 600 MHz. The chemical shift was measured in δ (ppm), and the coupling constant (*J*) was indicated in Hertz (Hz).

The antioxidant activity test was performed utilizing the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) assay using Trolox (a water-soluble vitamin E analog) as a positive control.

Extraction and isolation

For each sample, fruits, seeds, and leaves were cut into small pieces and dried separately, and then ground to obtain a coarse powder. The weight of each sample was 6 kg of fruits, 3 kg of seeds and 4.5 kg of leaves. Each sample was macerated using methanol 3x 24 hours at room temperature. For the extraction, 15 L of methanol was used for the fruits, 10 L for extracting the seeds and 12.5 L for the leaves. Each sample's methanol extract was separated using n-hexane, dichloromethane, and ethyl acetate successively. Monitoring each extract and fraction obtained by TLC provided an overview of the compounds contained so that it can be determined which extract or fraction has the potential to be separated to obtain the pure compounds. For the fruits and seeds samples, the ethyl acetate fraction was selected with the extract weights of 50 g and 98 g, respectively, while for the leaves, the dichloromethane extract was selected (9 g).

The ethyl acetate extract of the fruits was separated using VLC twice successively to obtain a pure compound, namely Compound-**1**. The first VLC was performed using an eluent of *n*-hexane-ethyl acetate, while the second VLC on one fraction obtained from the first VLC was carried out using an eluent of dichloromethane-ethyl acetate. Another fraction from the first VLC was re-separated using CC until a pure compound was obtained, namely Compound-**2**. The CC was performed using an eluent of n-hexane-ethyl acetate.

The ethyl acetate extract of the seeds was separated utilizing CC, which was eluted using *n*-hexane-ethyl acetate as eluent. The eluent polarity was gradient increased. Compound-**3** and Compound-**4** were obtained from this separation, and each was eluted with ethyl acetate.

The dichloromethane extract of the leaves was separated utilizing VLC with *n*-hexane-dichloromethane as eluent. One of the fractions obtained from this separation formed a precipitate and then recrystallized using methanol (Compound-**5**).

ABTS assay

The ABTS method tested the five isolated compounds for antioxidant activity. One mg of the pure compound was dissolved in 1000 mL of methanol to form a solution of 1000 ppm, which was then diluted to form a series of concentrations of 500, 250, 125 and 62.5 ppm. A total of 10 µL of samples with a particular concentration as stated above was added to 200 µL of ABTS reagent and 10 µL of buffer solution (pH 4.5), incubated for approximately seven minutes, and the absorbance was measured at λ_{max} of 734 nm. The % inhibition was calculated according to the following formula:

$$\%\ inhibition = \frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}}$$

Note: Abs blank is absorbance of solution without sample
Abs sample is absorbance of sample solution

Next, a curve of the relationship between % inhibition and sample concentration was made. The antioxidant capacity which is the ability to reduce ABTS radicals, was expressed in IC_{50}, which is the concentration at 50% inhibition. IC_{50} of Trolox as a positive control was also obtained.

Statistical analysis

Analysis of antioxidant activity testing of compounds 1 – 5 was carried out using an independent t-test with an error rate of 5%. This test was used to compare the antioxidant activity of isolated compounds (1-5) with Trolox as a positive control.

Results and Discussion

Compound-**1 :** white solid, 266 mg, IR spectrum (ν cm^{-1}) : 3470 and 3425 (O-H stretching), 2961 (C-H sp^3 stretching), 1740 (O=C-O); 1443 (C-H sp^3 bending), 1302 and 1128 (C-O ether); ^{1}H-NMR (MeOD) δH (ppm) : 2.82 (2H, *d*, *J* = 15.4 Hz), 2.94 (2H, *d*, *J* = 15.4 Hz), 3.66 (*s*, 2x OCH_3); ^{13}C-NMR completed with DEPT experiment δC (ppm) : 44.0 (CH_2), 52.2 (2x CH_3), 74.2 (C), 171.8 (2x C), 176.4 (C).

Compound-**2** : white solid, 131 mg, IR spectrum (νcm^{-1}) : 3470 and 3425 (O-H stretching), 2961 (C-H sp3 stretching), 1740 (O=C-O); 1443 (C-H sp3 bending), 1302 and 1128 (C-O ether); ^{1}H-NMR (MeOD) δH (ppm) : 2.79 (2H, *d*, *J* = 15.4 Hz), 2.94 (2H, *d*, *J* = 15.4 Hz), 3.66 (*s*, 2x OCH_3); 3.76 (s, OCH_3);^{13}C-NMR completed with DEPT experiment δC (ppm) : 44.3 (2x CH_2), 52.2 (2x CH_3), 53.1 (CH_3), 74.7 (C), 171.7 (2x C), 175.1 (C).

Compound-**3** : white solid, 24 mg, UV-Vis spectrum (MeOH) : λ_{max} 268 nm; IR spectrum (νcm^{-1}) : 3356 (O-H stretching), 3018 (C-H aromatic stretching), 2962 (C-H sp^3 stretching), 1693 (O=C-O), 1620 and 1537 (C=C aromatic), 1407 (C-O ether); ^{1}H-NMR (MeOD) δH (ppm) : 3.81 (*s*, OCH_3), 7.04(*s*, 2 identical aromatic CH); ^{13}C-NMR completed with DEPT experiment δC (ppm) : 52.7 (CH_3), 110.5 (CH), 169.4 (C), 140.2 (C), 146.9 (C), 121.9 (C), 146.9 (C).

Compound-**4** :white solid, 10 mg, UV-Vis spectrum (MeOH) : λ_{max} 272 nm; IR spectrum (ν cm^{-1}) : 3229 (O-H stretching), 2932 (C-H aromatic stretching), 2664 (C-H sp^3stretching), 1694 (O=C-O), 1616 and 1545 (C=C aromatic); ^{1}H-NMR (MeOD) δH (ppm) : 7.06 (s, 2 identical aromatic CH);^{13}C-NMR completed with DEPT experiment δC (ppm) : 110.7 (CH), 170.9 (C); 146.8 (C); 139,9 (C); 122.6 (C); 146.8 (C).

Compound-**5** : white solid, 82 mg, UV-Vis spectrum (MeOH) : λ_{max} 289,5 and 260,5 nm; IR spectrum (ν cm^{-1}) : 3435 (O-H stretching), 3072 and 3011 (C-H aromatic stretching), 2955 (C-H sp^3stretching), 1742 (O=C-O), 1610 and 1489 (C=C aromatic), 1360 (C-H sp^3bending), 1242, 1167 and 1101 (C-O ether); ^{1}H-NMR (DMSO d6) δH (ppm) : 7.57 (1H, *s*), 7.49 (1H, *s*), 4.05 (*s*, OCH_3), 4.03 (*s*, OCH_3), 3.98 (*s*, OCH_3); ^{13}C-NMR completed with DEPT experiment δC (ppm) : 57.2 (CH_3), 61.3 (CH_3), 61.7 (CH_3), 107.9 (CH), 112.3 (CH), 111.2 (C), 112.2 (C), 112.8 (C), 113.9 (C), 140.8 (C), 141.1 (C).(C), 141.4 (C), 141.8 (C), 153.6 (C), 154.1 (C), 158.7 (C ester) 158.9 (C ester).

UV-Vis spectrum in methanol of Compound-**1** did not give an absorption in the region above 200 nm. This indicates that Compound-**1** does not have any conjugated systems. The IR spectrum proved the existence of a hydroxyl group, C-H $sp^3$and ester group. Analysis of the ^{1}H-NMR spectrum showed two proton signals that appeared at δH 2.82 (2H, *d*, *J* = 15.4 Hz) and at δH 2.94 (2H, *d*, *J* = 15.4 Hz). According to the HSQC spectrum, these four protons are bonded to the same C atom, so they were two identical methylenes (CH_2). This spectrum also gave a proton signal at δH 3.66 (6H, s), which was correlated with one carbon signal in the HSQC spectrum, so that it was known as two identical methoxy groups (OCH_3). The presence of the hydroxyl group was confirmed here with a signal that appeared at δ 74.2 ppm. This compound also had two ester groups and one carboxyl group. A comparison with the literature[11] confirmed that Compound-**1** was 2-hydroxy-4-methoxy-2-(2-methoxy-2-oxoethyl)-4-oxobutanoic acid or 1,5-dimethylcitrate. The structure is shown in Figure 1.

The difference in the NMR spectra of Compound-**1** and Compound-**2** lied in the number of methoxy groups. Compound-**1** had two, while Compound-*2* had three, therefore, Compound-**2** was identified as trimethyl-2-hydroxypropane-1,2,3-tricarboxylate or trimethyl citrate. The structure of this compound is shown in Figure 1.

Table 1: Result of ABTS Assay

Compounds	IC_{50} (µg/mL)
Compound-**1**	1.421
Compound-**2**	836
Compound-**3**	90.6
Compound-**4**	29.6

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Compound-**5**	911
Trolox	77.4

Simple NMR spectra of Compound-**3**, both ^{1}H and ^{13}C, indicated the presence of a methyl ester group and two identical aromatic methines that appeared as singlets. Thus, the compound has a tetrasubstituted benzene fragment. Since the IR spectra indicated the presence of a hydroxyl group, Compound-**3** was expected to be an aromatic polyhydroxy compound, namely; methyl ester of gallic acid. This information was confirmed by UV spectrum data, which gave an absorption in the phenolic compound range. All the spectra data of this compound were compared with literature.[12] The structure of Compound-**3** is shown in Figure 1.

The NMR spectrum of Compound-**4** showed the same information as Compound-**3** except for the loss of the methoxy signal. Thus, it can be concluded that Compound-**4** was gallic acid. The structure is shown in Figure 1. The UV-Vis spectrum of Compound-**5** showed two λ_{max}; 289.5 and 260.5 nm, which corresponded to the phenolic compounds. The ^{1}H-NMR spectrum depicted the existence of five proton singlet signals, consisting of two signals at δ7.57 and 7.49 ppm, which indicated the presence of two aromatic protons and three signals at 4.05, 4.03, and 3.98 ppm, which indicated the presence of three methoxy groups. ^{13}C-NMR spectrum analysis equipped with DEPT 135 and DEPT 90 showed the presence of 17 carbon signals, consisting of ten quaternary carbon signals, two aromatic methine signals, and three carbon signals of methoxy and two carbon signals of ester groups. Based on the spectroscopic data analysis, it was confirmed that the Compound-**5** was 3,4,3'-tri-*O*-methylellagic acid, the structure of which is shown in Figure 1. This compound has also been reported to be isolated from the chloroform extract of the bark of *S. polychepalum.*[7]

Based on the ABTS assay results shown in Table 1 above, only Compound-**4** (gallic acid) showed strong antioxidant activity and was better than Trolox. Compound-**3** also showed antioxidant activity, although its activity was lower than that of Trolox, while the others did not show antioxidant activity. All extracts obtained from maceration and partition were also tested for antioxidant activity. Only ethyl acetate extract of the seeds showed weak antioxidant activity. Other extracts did not show antioxidant activity.[13] The results of the t-test showed that there was no significant difference between the antioxidant activity of the isolated compounds (1-5) and Trolox as a positive control, because the t-tailed value was > 0.05.

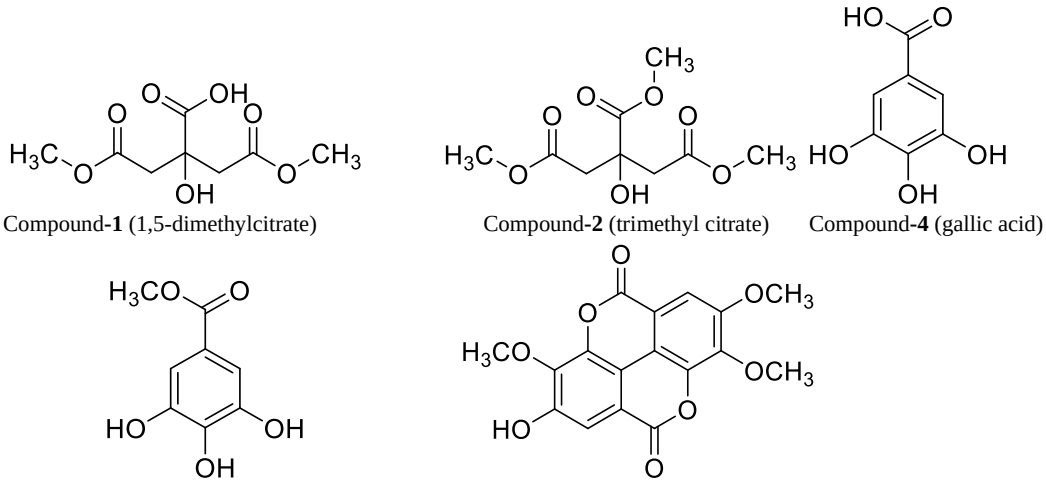

Figure 1: Structure of isolated compounds

Conclusion

Antioxidant bioactive compounds from the fruit, seeds, and leaves of *S. polycephalum* have been identified as 1,5-dimethylcitrate, trimethyl citrate, methyl gallate, gallic acid, and 3,4,3'-tri-O-methylellagic acid. The antioxidant test using the ABTS radical scavenging assay showed that gallic acid had better antioxidant activity than the control, Trolox.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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